Masters of Research

Genetic diversity and structure of Moreton Bay fig (*Ficus macrophylla*) in mainland Australia and Lord Howe Island

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Statement of authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Contribution Statement

The statement is organised following the structure of the thesis paper as follows.

Sampling

The source materials for the thesis project were the archived samples from Desi Quintans Honours project used for genotyping, the samples I collected from the Royal Botanic Gardens which were used for the majority of the project and samples collected by Jane De Gabriel and Ben Moore during their travels to Lord Howe Island.

Genotyping

A total of 18 markers were used in the thesis project. I developed the majority of markers by myself with some assistance from my supervisors during the process. These were 1_03Fb, 1_11Fg, 1_14Fr, 1_23Fy, 2_01Fr, 2_02FrA, 2_02FrB, 2_03Fg, 2_05Fb, 2_06Fg, 2_15Fr, 2_20Fb, 2_33.

The other five markers used were developed by Desi Quintans during his Honours thesis. These were Micr 1, frub 29, frub 61, frub 415, frub 436.

Furthermore, I genotyped the source material used (Desi's Honours samples) with my markers. Then I genotyped my samples collected from the Royal Botanic Gardens Sydney using all the markers (Desi's and mine) to increase the existing dataset created by Desi Quintans.

Genetic analyses

All the genetic analyses were done by myself under the supervision of my supervisory panel. In addition, these analytical tools were used as stated in the

methodologies and were recommended by my supervisory panel as the most appropriate tools for the application.

Flowering phenology

The flowering phenology of the Moreton Bay fig trees was surveyed by myself on a fortnightly basis for 6 months at Royal Botanic Gardens for the period August 2017 to February 2018. My supervisor Paul Rymer reviewed and provided comment on the analysis of the surveys when the data was finally collated onto a spreadsheet.

Seed viability

For the seed viability component I used fig fruits I collected from the Royal Botanic Gardens Sydney in November 2016 and the material provided by Jane De Gabriel and Ben Moore during their travels to Lord Howe Island.

I germinated the fig seed in pots with soil containing slow release fertiliser and were left in the shade house at Hawkesbury campus. I then over 18 months observed the growth of each pot making notations and counting the final total number of seedlings per pot and analysed the data. In addition, the source material that grew from the Lord Howe Island fig fruit was used in the genotyping experiments.

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I would like to thank my thesis supervisors Paul Rymer and Collin Aherns and James Cook of the Hawkesbury Institute of the Environment for their guidance and support and consistently steered me in the right direction and providing comment on my work.

Furthermore, I would like to acknowledge John Martin from the Sydney Royal Botanic Gardens for facilitating access to the precinct and its fig trees for sampling.

I would also like to thank Jane De Gabriel, Ben Moore, Desi Quintans and James Cook and Seth Meyers for providing the samples that I was able use in this project. Marcus Klein for his invaluable technical expertise in genotyping and sequencing which was key to my laboratory work.

Finally, I must express gratitude to all the administrative and technical staff of the Hawkesbury Institute of the Environment for their support through the process of researching and writing this thesis.

Abstract

Genetic contamination of natural population through human dispersal has the potential to erode natural genetic diversity through competition or introgression. The Moreton Bay fig (*Ficus* macrophylla) has two distinct forms, *macrophylla* found in eastern Australia and *columnaris* in Lord Howe Island (LHI). Given the world heritage status, high levels of endemism and unique biological processes on Lord Howe Island preventing genetic contamination is an important environmental, social and economic issue.

This project aims to increase the understanding of the potential for genetic contamination to occur among the two forms, including determining how genetically distinct two forms are and whether they can produce viable hybrid offspring.

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Introduction

Background

Across the Ficus macrophylla population there is a continuum of individuals within various smaller populations across the large distribution of the Australian mainland and Lord Howe island. These populations are interconnected but are genetically distinct populations and the Lord Howe island is a reproductive isolated population.

Over time there are processes that have driven and eroded the genetic diversity and population size of the species. These processes have included: genetic drift, mating patterns, geneflow, selection and mutation.

Genetic diversity

Genetic diversity refers to the variety of alleles and genotypes present in a population, species or group of species (Frankham et.al, 2007). It is critical to understand the variety of alleles and genotypes of a population, species or group of species as the mechanism by which they are able to continually adapt to the environmental changes that may occur and to reduce the potential for inbreeding within populations (Frankham et.al, 2007). In practice the use of molecular techniques is required to measure genetic diversity. These molecular techniques use DNA or soluble proteins which uses allozymes, microsatellites to amplify certain sections of the sequence to identify the genotypic differences among the population or species (Frankham et.al, 2007).

Population Genetics

Population genetics is a mathematical model to explain Charles Darwin's theory of evolution and Gregor Mendel's laws of inheritance (Dronamraju, 2016). This major

contribution was made by J.B.S. Haldane, R.A. Fisher and S. Wright. Key applications of this theory in society include improving crop and animal breeding methods, epidemiology of diseases and defects in populations and the genetic basis of kinship (pedigrees) (Dronamraju, 2016). This mathematical model also contributed to the development of the standardised null hypothesis model called the Hardy-Weinberg equilibrium that can enable the quantification of allelic frequencies and genotypic frequencies (Dronamraju, 2016). Changes to this equilibrium model provide a way to confirm whether the study population is experiencing non-random mating, migration or selection (Dronamraju, 2016, Frankham et.al, 2007). Furthermore, these factors that are used in the equilibrium affect the population size and the abundance and distribution of genetic diversity. They are categorised as natural factors and act upon all levels of ecological hierarchy which occur in varied frequencies over a long or short period of time. On the other, the actions and influence of humanity has affected the demography of plant species across the world via forest clearing and propagation of plant species away from their natural distribution. As a result, these actions can lead to species experiencing greater pressure to adapt to changes to the environment and in the process alters the genetic diversity resulting in speciation or hybridisation.

In addition, the effect of these factors whether human or natural affects the population size and genetic diversity therefore can result in an additional consequence called inbreeding. Inbreeding is caused when there is a reduction of genetic diversity, increase in homozygosity and small population size. Inbreeding is measured using F statistics and it involves comparing the level of inbreeding between the individual and the subpopulation (Wright's inbreeding coefficient - F_{is}),

Individual and the overall population (F_{it}) and the subpopulation and the overall population (F_{st}).

Therefore, by applying the principles of population genetics in an important Australian fig species I can identify patterns of neutral genetic structure, quantify gene flow between populations and summarise potential genetic contamination.

Theory of Island Biogeography

The theory of island biogeography explains how much speciation occurs on an island environment based on the interaction between the number of extinctions of species and the quantity of migration of species to the island environment (MacArthur, Osborne, 1967). As a result, the equilibrium of this interaction can be affected by:

- the distance between the island environment and the nearest landmass,
- length of isolation
- the size of the island
- human activity

The distance between Lord Howe island and the Australian mainland affects the possible amount and rate of species that migrate onto the island as well as the level of genetic diversity and gene flow that occurs between the two landmasses. In addition, the length of time Lord Howe island has been established which is approximately 6.4 to 6.9 million years ago, have given an opportunistic moment for species from the Australian mainland to establish and evolve over time to become a new distinct species from its original species. The next key factor is the size island. The size of the island affects how many species the island can have at any one time due the amount of food and viable habitats there are available for species to flourish before succumbing to extinction (MacArthur, Osborne, 1967). Furthermore, other

elements that affect an island's genetic diversity are the climatic conditions, the existence of a previous ancestral population and the impact of human activity.

Lord Howe Island has been well established with an abundant genetic diversity and biodiversity while having an established accessibility to gene flow from mainland Australia (Savolainen et.al, 2006).

Speciation

Speciation is the evolutionary process that explains why distinctiveness of species occurs at a single location and how the existing species and the neighbouring populations are connected (Mason et.al, 2014). Furthermore, the concept of speciation can be broken down into different geographic speciation scenarios, these include:

Allopatric – when populations are isolated by an external barrier and develop intrinsic reproductive isolation such that if the barrier breaks down, the populations cannot interbreed (Biology-online.org, 2018)

Peripatric – A new species forms in a sub-population that inhabits a new environment within the same geographical area of the ancestral species (Biology-online.org, 2018)

Parapatric –two diverging populations are partially separated however contact can be made on occasions until specific behaviours or isolation mechanisms inhibit the two populations from interbreeding. (Biology-online.org, 2018)

Sympatric – the differentiation of populations within a common geographic area into species (Mason et.al, 2014).

These modes of speciation are important because they explain how the genetic diversity splits and forms a new species from an existing population. In addition,

there are mechanisms which can be observed and can explain how genes within each of these scenarios are exchanged. These mechanisms include: geographical isolation, ecological isolation, mechanical isolation, behavioural isolation, temporal isolation, prevention of gamete fusion and hybrid infertility and in viability (Mason et.al, 2014).

Furthermore, the two key mechanisms in this research study are hybridisation and geographical isolation. Hybridisation commonly occurs in plants and animals. Botanists and zoologists have different views of hybridisation due to how it is seen to affect evolution. Botanists believe it is an important mechanism for production of new species and novel adaptations (Allendorf et.al, 2013). In contrast, zoologists believe the role of hybridisation results in offspring that are often relatively unfit that leads to reproductive isolation and speciation. (Allendorf et.al, 2013).

Geographical isolation is a mechanism that results in an ancestral population diverging into different geographic regions that then reproduce in isolation and develop different characteristics that end up creating a unique species (Mason et.al, 2014).

Context

The Moreton Bay fig (*Ficus macrophylla*) occurs naturally from central Queensland to southern New South Wales and on the World Heritage Site, Lord Howe Island. The island is approximately 600km offshore from Port Macquarie New South Wales and was created approximately 6.4 to 6.9 million years by the activity of nine underwater volcanoes (Savolainen et.al, 2006). As a result, it has given rise to unique and endemic species found exclusively on Lord Howe Island and consequently has

created interest in better understanding of sympatric speciation (Savolainen et.al, 2006).

The Moreton Bay fig exists in two forms, the Australian mainland form (*Ficus macrophylla macrophylla*), a singular free-standing trunk and the Lord Howe Island form (*Ficus macrophylla columnaris*), a buttressing root system called “banyans” (Dixon, 2001). However, given the morphology of leaves and fruits are similar, and the two forms share the same fig-wasp pollinator (*Pleistodontes froggatti*) the mainland and Lord Howe Island forms haven’t been elevated to subspecies level (Dixon, 2001).

The Moreton Bay fig is a key species among the Australian ecosystem because it plays a major role in providing Australian fauna with food provisions when other fruits are not in season. As a consequence, the seed from the fruit has the potential to be dispersed over long distances and new locations during the process of digestion and defecation. In addition, the fig wasps can travel several hundred kilometres via wind dispersal to transport pollen from one fig tree to another.

Even though these two forms share a common pollinator, their phenologies may create a barrier to gene flow. Currently phenological surveys have recorded the stages of the fig life cycle over an extended period of time and it is possible to identify at what times of the year the fig wasp (*Pleistodontes froggatti*) are able to access receptive fig fruits for pollination of both forms of *Ficus macrophylla* (Jia et al. 2008, McPherson, 2005). However, these were surveys were about the reproductive cycles of the fig wasp (*Pleistodontes froggatti*) and its related species rather than the reproductive cycle of the fig (Jia et al. 2008, McPherson, 2005).

Recently, the topic of genetic contamination has been raised in relation to the maintenance of Lord Howe Island’s Moreton Bay fig genetic diversity. Two planted

Moreton Bay figs with the mainland characteristics have been identified and National Parks rangers have requested genetic verification prior to removal. Therefore, identifying the phenological overlap and reproductive effort of the Moreton Bay fig while determining the success and potential presence of hybrids associated with human plantings.

Furthermore, the Lord Howe Island local government implemented 'The Weed Management Strategy' in an effort to control possible contamination of the *Ficus macrophylla columnaris* population by the *Ficus macrophylla.* The *Ficus macrophylla* is categorised as an alert species (sleeper weed) that has the potential to hybridise with the island population with possible loss of genetic diversity. It is currently unknown whether genetic contamination has occurred because it has not yet been confirmed whether the plant individuals of each form have reproduced with each other. Until now there has only been a hypothesis that the *Ficus macrophylla columnaris* and the *Ficus macrophylla macrophylla* could have hybridised but there is yet any conclusive evidence of the existence of ongoing hybridisation between the two forms.

Therefore, the aim of this study is to provide a better description of the structure and differentiation of genetic variation within the Moreton Bay fig. therefore, this thesis aims to elucidate whether the mainland and Lord Howe island populations are genetically distinct? Does gene flow occur between mainland and Lord Howe island populations? And what is the impact of the planted material on the integrity of Lord Howe Island?

This project will investigate the following hypotheses:

- There is greater genetic diversity in the mainland populations compared to the Lord Howe island population

- Mainland and Lord Howe island populations are genetically distinct
- Reduced gene flow from the mainland to/from Lord Howe island
- Lord Howe island has reduced effective population size with greater inbreeding compared to mainland populations
- Reduced phenological overlap among the mainland and Lord Howe island forms compared to overlap of the reproductive stages within the forms

Methods

Sampling

This sampling design involved collecting plant material from naturally occurring trees that represented most of the geographical distribution of the species and which met one or more of the following criteria: being 150 years old or more with known lineage, grown as stranglers, emergent tree from an old growth forest, a remnant tree on pasture land or local source material planted by bushland regenerators (Quintans, 2014). The four locations these trees were sampled include Illawarra, Lismore, Lord Howe Island and Coast (between Illawarra and Lismore). A total 204 trees were sampled from these four locations and the samples were stored and dried with silica beads (Quintans, 2014). The locations Illawarra, Lismore, Lord Howe Island were more densely sampled with the intention of providing power in estimating allele frequencies and observed heterozygosity. The coast was sampled in a random stratified method at intervals of 50 to 150 kilometres to ensure adequate representation over the large distance between the locations Illawarra and Lismore (Quintans, 2014). Leaves from the selected individual figs were collected when available otherwise a cambium sample was collected of the desired individual fig using a 10mm diameter hollow leather punch. The leaves obtained were mature leaves, deep green in colour and still turgid in structure. In this study a subsample of 114 from the four locations was created (31 for Illawarra, 30 for Lismore, 33 Lord Howe Island and 20 coastal) (Quintans, 2014).

Genotyping

The CTAB extraction protocol was used to extract DNA from cambium or leaves of individual fig trees (Doyle and Doyle, 1987). Minor modifications were made to the

protocol to deal with the latex content of the *Ficus* tissue. These modifications included adding an extra 200uL of CTAB buffer and an extra chloroform: isoamyl alcohol extraction (steps 9 to 12 of the protocol). The extracted DNA was stored in 100ul of TE buffer or DNase/RNase free water at -20 °C.

Multi-locus PCR (polymerase chain reaction) amplification was performed using a QIAGEN Multiplex master mix (QIAGEN #206145) per the manufacturer's recommendations. The PCR reactions volumes were 10 µL per sample with 1 µL of 1:20 DNA (concentrations standardised) and 0.1uL of Forward primer and Fluorophore and 0.2uL Reverse primer at 10 pmol concentration. The Hot-start annealing Cycling conditions used were 95 °C denaturation for 2 minutes, 30 cycles of 94 °C melt for 30 seconds, 60 °C annealing for 1 min, and 72 °C extension for 1:30 minutes, with a final 72 °C extension period for 10 minutes followed by a 4°C cool down. All PCR thermocycling was performed on a Biorad Dyad System thermocycler.

Fragment analysis was carried out on an Applied Biosystems 3500 Genetic Analyser with the addition of the LIZ600 internal size standard. The fragment analysis output data was visualised in the program GeneMapper 5 (v5.0 Applied Biosystems) using an automated allele binning function which used the information about the base pair range and the repeat motif of each microsatellite used. The automated allele binning function then uses the information created to score the intensity (height) and the size (number base pairs) of each peak that appeared for each sample that was run with its corresponding fluorescent marker. In addition, the results for the automated function were also checked manually for any incorrect selection of alleles.

Genetic analyses

The data was then imported into the program Microchecker (Van Oosterhout et al., 2004) to determine the quantity of existing null alleles in each of the four populations sampled for this experiment. In addition, the program uses four statistical methodologies to calculate the frequency of null alleles within the dataset. The methodologies are named after the authors who created it; Oosterhout, Chakraborty, Brookfield 1 and Brookfield 2. These four statistical methodologies vary in their calculations because it depends whether the samples have failed to amplify or whether the nonamplified samples are null homozygotes. Furthermore, this is an indication of null alleles are a result of a significant number of observed homozygotes within the highlighted loci.

GenAlEx (a Genetic Analysis cross-platform package for population genetic analyses that runs within Microsoft Excel) (Peakall et.al, 2012) was used to conduct frequency and distance-based analysis. The analyses performed were F-statistics and the observed heterozygosity was also calculated. The F-statistics are used to identify the quantity of inbreeding within the population, amongst the individuals and between populations. In addition, the observed heterozygosity is used to identify the quantity of heterozygosity in the population.

STRUCTURE analysis was run to identify patterns of population structure from the individuals sampled. The specific parameters were run length: 10000, burn in MCMC: 10000, K=1-4 with 5 repetitions of each K and the ancestral model used was admixture and allele frequency were correlated. The Evanno method is used to find the K-value (Evanno et.al, 2005). The visual representation of the STRUCTURE output firstly began by collating the multiple runs for each K value using CLUMPP and then Distruct was used to create Figure 2 showing individual bar graphs

partitioned according to the estimated relation to each of the K clusters (Jakobsson et.al, 2007, Evanno et.al, 2005, Rosenburg, 2003).

In addition, a discriminant analysis of principal components was performed using an R script on R. The R function uses the input data to perform discriminant analysis of principal components is performed using the retained principal components (Jombart et al. 2015). In this study, the parameters used in the R script were 20 principal components were retained and 2 discriminant components were retained (Jombart et al. 2015). Following these steps, a scatter plot is produced to visually represent the results produced by the analysis. In addition, to the graph a summary can also be produced (Jombart et al. 2015).

To assess the evolutionary history and demographic processes shaping the mainland and Lord Howe Island populations a suite of analyses was performed.

BayesAss is designed to determine the amount and direction of ‘recent’ gene flow among populations (Wilson, Rannala, 2003). Using the mixing parameters, the methodology was optimised to ensure an accurate convergence of the population data. The parameters set for each were the except the seed number which changed twice. The parameters were as follows, delta A - 0.3, delta F - 0.3, delta M - 0.2, iterations - 10000000, burnin - 1000000, sampling - 100, seed number - 113, 100,1000 and input file (Wilson, Rannala, 2003).

DIYABC is a program that identifies the possible splitting time among the current study populations (Cornuet et.al, 2014). The program uses summary statistics to replace raw observed data inputted to the program (Allendorf et.al, 2012). Then the program computes these summary statistics for each iteration of simulated dataset and matches these results with the observed summary statistics to choose the population parameter estimate that best fits the data (Allendorf et.al, 2012).

Therefore, for this study 4 scenarios were modelled. The four scenarios modelled were as follows:

Table 1 Potential evolutionary scenarios tested with the DIYABC model

Scenario	First event (t2)	Second event (t1)
1	Lord Howe Island split from Lismore	Illawarra split from Lismore
2	Lord Howe Island split from Illawarra	Lismore split from Illawarra
3	Illawarra split from Lord Howe Island	Lismore split from Lord Howe Island
4	Lismore split from Lord Howe Island	Illawarra split from Lord Howe Island

The historical model for the scenarios assigned the northern and southern mainland populations with a population size of 100000 and the Lord Howe island population with a population size 40000 (Cornuet et.al, 2014). The splitting events were assigned after 10000 generations and assigned the condition that the second splitting event could not be larger than the first splitting event (Cornuet et.al, 2014).

Flowering phenology

The survey was performed according to the methodology presented by Jia et al. (2008) however, it was modified to fit within the time constraints of the research project. A survey was carried out every fortnight for a period of 24 weeks on Moreton Bay fig trees within the Royal Botanic Garden precinct in Sydney while taking note of the developmental phases A–E of a high and low branch on each of the four cardinal points on the tree. The aim of the survey was to identify presence of each of the developmental phases A–E and focusing especially on the possibility of B and D

phase overlap within a tree, which provides an opportunity for self-pollination and hybridisation events. The reason for focusing on phases B and D was because as shown in Figure 3 is when the pollinator *Pleistodontes froggatti* deposits the pollen (phase B) and emerges from the fig fruit prior to the full maturity (phase D). Therefore, it is especially important if the pollen has come from the Lord Howe Island form because this results in the transfer and exchange of genes from one form to the other.

The data was collated and analysed on Microsoft excel. Once the data was entered into the excel spreadsheet, the data was combined and sorted according to the following categories; day of the survey process, calendar date, form of Ficus, sampled tree, cardinal point, high or low branch, branch number and chronological order of fig stages. This data then was used to calculate the average and standard deviations of stages B and D followed by calculating the potential of self-pollination, outcrossing and hybridisation in terms the proportions of B and D within the same tree and the proportions of B and D ((max-difference)/max).

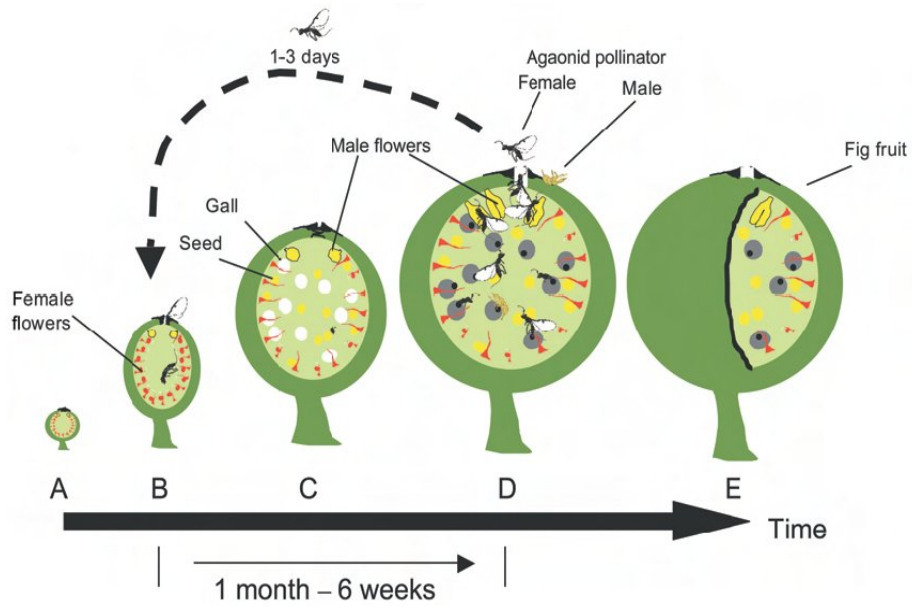

Figure 1 Development stages in Ficus macrophylla (Cranston and Gullan, 2010)

Seed viability

The fig fruit were collected from the Royal Botanic Gardens Sydney in May 2016 with the assistance of John Martin. In addition, samples were also collected from Lord Howe Island by Jane de Gabriel and Ben Moore. These samples were all stored in silica to remove excessive moisture that can cause mould to develop. Once these samples were ready the seed were remove from the husk and were planted in all purpose potting mix with slow release fertiliser. The sample sizes for each fig form was 70 for the mainland, 26 for the Lord Howe island (Royal Botanic Gardens) and 22 for Lord Howe island. Once the seeds were planted the pots were placed in a shade house facility with automated sprinkler system for irrigation. The seedlings were monitored on a fortnightly basis to remove weeds and to observe the quantity of emergence. This monitoring continued over the next 18 months till a definitive number of emerged seedlings were able to be counted.

The seedlings that had emerged from each individual fig fruit was recorded. The data was then inserted into an excel spreadsheet and the mean and standard errors were calculated.

Results

Molecular and ecological datasets were collected and analysed to determine the level of genetic diversity, structure, migration, population size and splitting times among *F. macrophylla* populations from the mainland and Lord Howe island populations.

Genetic diversity

A total of 18 nuclear microsatellite markers were genotyped for 114 adult trees from across the geographic distribution of *F. macrophylla*. Three markers were removed from further analysis, because of missing data due to poor amplification, which is an indication of null alleles. 21 individuals had greater than 50% of missing markers were removed from the analysis. The remaining 93 individuals genotyped for 15 markers averaging 16% missing data among all markers (25% had less than 6% missing data; 75% of the population had greater than 20% missing data) overall variation in the 15 SSR markers had a minimum of 5 alleles and maximum of 11 alleles (mean 7.2 +/- 0.5 SE)

The Microchecker analysis highlighted loci 1_03fb, micr1, 1_23fy, 2_15fr, 2_33, frub415 and 2_01 with possible null alleles, and deviation from Hardy Weinberg equilibrium. The 1_03fb, micr1, 2_33, and frub415 were highlighted with potential null alleles in all three populations (Illawarra, Lismore, LHI). In addition, 1_23 and 2_15 were highlighted loci with possible null alleles in the Illawarra population and the Lord Howe island population had the 2_01 loci highlighted as a possible null allele. Microchecker is not sensitive to whether the null alleles have failed to amplify or

whether the nonamplified samples are null homozygotes. Therefore, these null alleles maybe a result of a significant number of observed homozygotes.

The levels of genetic diversity were different among the sampled populations. The Lord Howe Island population had lower levels of genetic diversity than the mainland populations with effective number of alleles detected in Lord Howe Island (1.81 +/- 0.19) was lower than that detected in Lismore (2.62 +/- 0.30), Coastal (2.66 +/- 0.27), and Illawarra (2.43 +/- 0.25). Similarly, Shannon's diversity index (Table 2) and the unbiased estimate of expected heterozygosity was lower in Lord Howe Island (0.39 +/- 0.05) compared to the mainland populations (0.57 +/- 0.05, 0.60 +/- 0.06, 0.53 +/- 0.06). Overall, there was no difference in the levels of genetic diversity detected in the three mainland populations.

Table 2 The mean and standard error of populations

Population	N		Ne		I		Ho		uHe		F	
LIS	17.64	(1.26)	2.62	(0.30)	1.02	(0.11)	0.40	(0.05)	0.57	(0.05)	0.23	(0.09)
CST	12.79	(0.94)	2.66	(0.27)	1.05	(0.11)	0.37	(0.06)	0.60	(0.06)	0.32	(0.10)
ILL	25.79	(0.54)	2.43	(0.25)	1.02	(0.11)	0.38	(0.06)	0.53	(0.06)	0.27	(0.07)
LHI	22.50	(1.33)	1.81	(0.19)	0.71	(0.10)	0.33	(0.07)	0.39	(0.05)	0.24	(0.12)
Total	19.68	(0.84)	2.38	(0.13)	0.95	(0.06)	0.37	(0.03)	0.52	(0.03)	0.26	(0.05)

N is the number of individuals samples. Ne is the number of effective alleles. I Shannon's index. Ho is the observed heterozygosity. uHe is the unbiased expected heterozygosity. F is the inbreeding coefficient.

The overall levels of observed heterozygosity were less than that expected based on Hardy Weinberg Equilibrium, such that the inbreeding coefficient for *F. macrophylla* was moderate (F = 0.26 +/- 0.05; Table 2). All three populations have reduced levels of heterozygosity (comparison between H_o and uH_e; Table 2), resulting in the inbreeding coefficient (F) being greater than 0. This indicates the system is not randomly mating, and there is evidence for partial self and/or biparental inbreeding (F

= 0.24 to 0.32) in all populations. The inbreeding coefficient in the Coastal sample was marginally elevated compared to the other populations, given adults were sampled from Sydney to Brisbane (~1000km) this may be due to non-random mating and population substructure (Wahlund Effect).

Genetic structure

The visual representation of the STRUCTURE output firstly began by collating the multiple runs for each K value using CLUMPP and then Distruct was used to create Figure 2 showing individual bar graphs partitioned according to the estimated relation to each of the K clusters (Jakobsson et.al, 2007, Evanno et.al, 2005, Rosenburg, 2003). The reason for showing Figure 2 in such manner was to provide a comparison between having 2 and 3 genetic distinct clusters. However, one irregularity that may affect the identification of genetic clusters through programs like STRUCTURE and other related ones is the concept of admixture. Admixture can affect how an individual sample can be sorted within the program without placing certain threshold to determine which genetic cluster the individual sample may belong to, furthermore the example of K = 3 provides evidence as to how the irregularity of genetic admixture can affect the decision of selecting one delta K value.

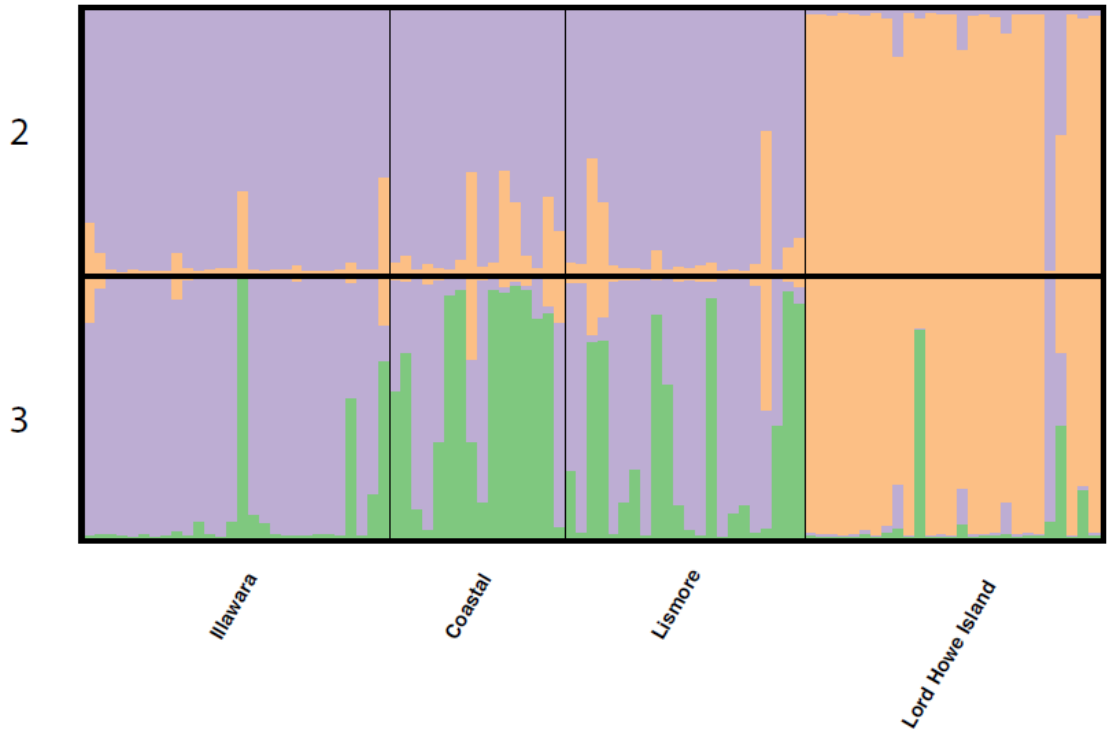

Figure 2 Bar plots from STRUCTURE constructed using Distruct and CLUMPP showing K=2 and K=3 showing the ancestral composition for each individual from mainland and LHI populations of Ficus macrophylla.

The reason for showing Figure 2 in such manner was to provide a comparison between having 2 and 3 genetic distinct clusters. However, one irregularity that may affect the identification of genetic clusters through programs like STRUCTURE and other related ones is the concept of admixture.

The genetic differentiation among populations estimated as Fst value from a molecular variance analysis was 0.188. While, the pairwise Fst values between the three populations were Illawarra and Lismore 0.103, Lord Howe Island and Lismore 0.162, and Lord Howe Island and Illawarra 0.219.

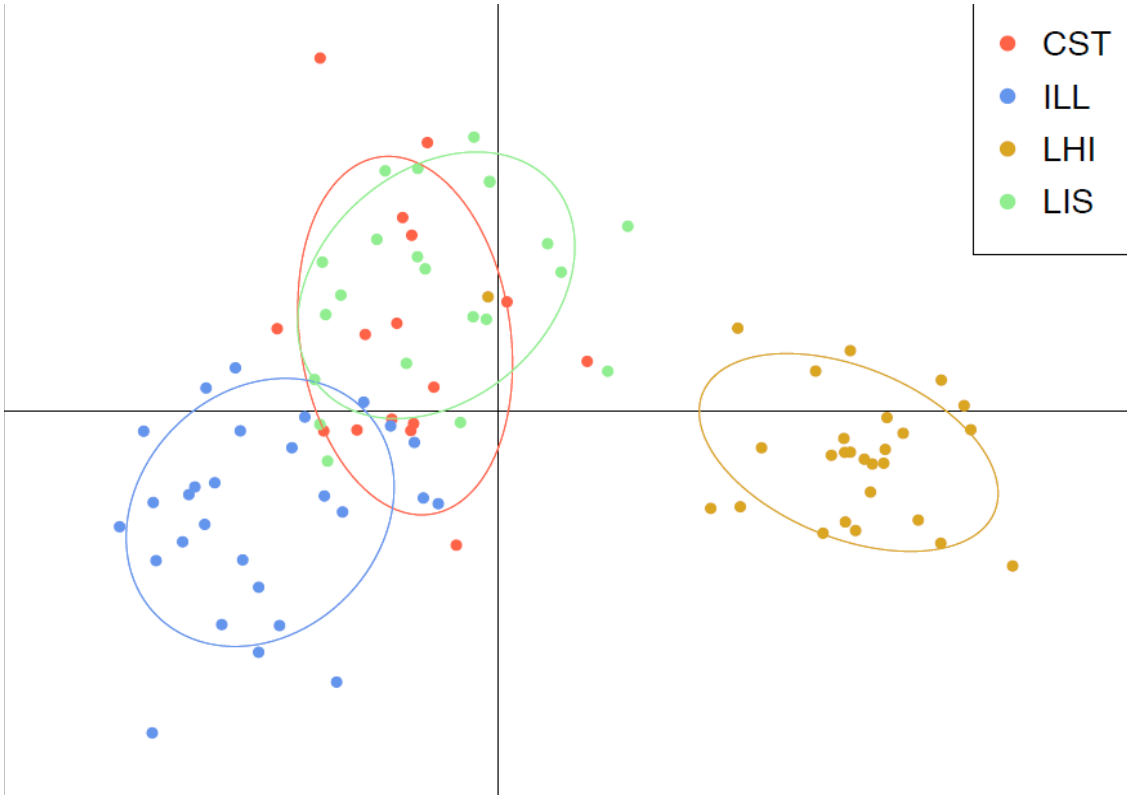

Figure 3 Discriminant Analysis of principal components (DAPC) of Ficus macrophylla populations from the Australian mainland (Illawarra, coastal, Lismore) and Lord Howe Island.

A discriminant analysis of principal components shows clusters of genetically related individuals clearly separating Lord Howe Island from the mainland populations. The mainland southern (ILL) and northern (LIS) populations are connected with a series of intermediate (CST) populations. The discriminant analysis of principal components explains 78.5% of the variation between the two axes (Figure 3).

Migration

The results from the BayesAss analysis illustrate that the greatest amount of gene flow is unidirectional towards Illawarra from Lismore. The level of gene flow to/from

Lord Howe island was an order magnitude lower. In addition, Figure 4 illustrates high significance in the amount of gene flow among the three major population areas to hypothesise that the northern mainland population (Lismore) is the origin point of the Moreton Bay fig species.

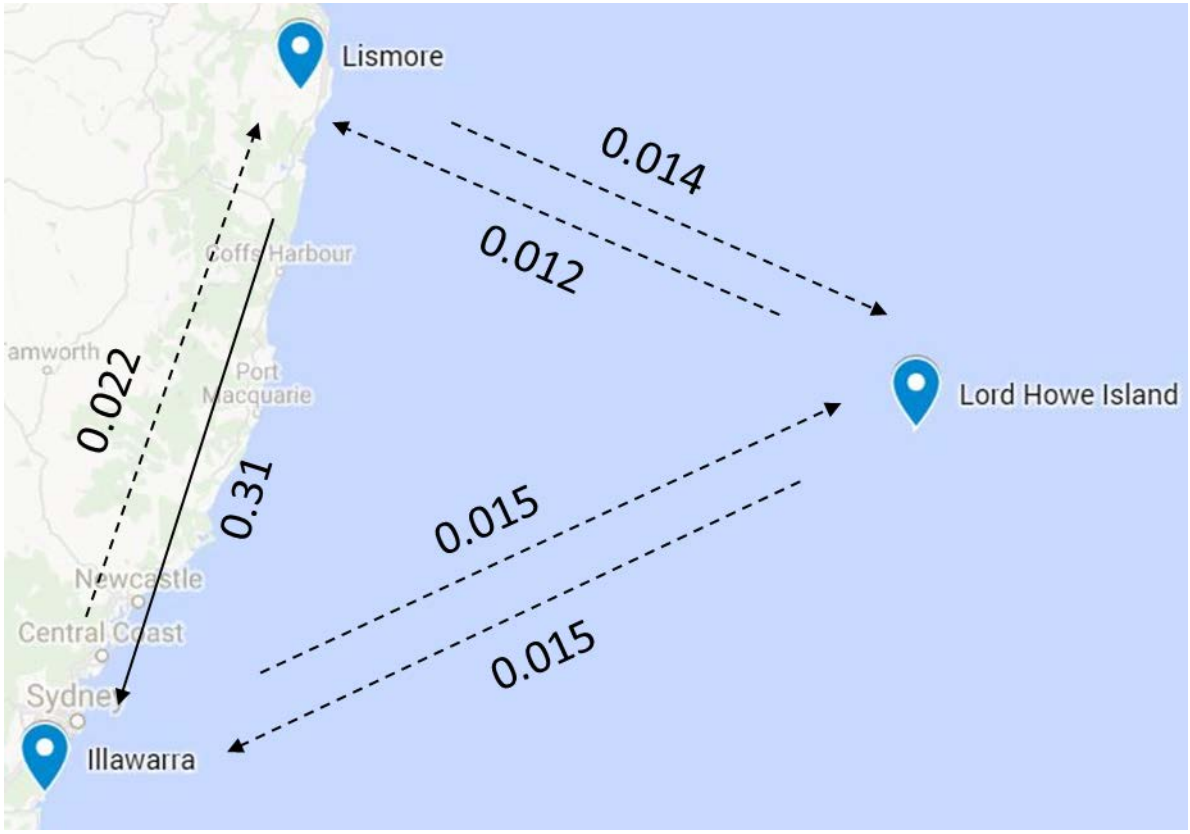

Figure 4 The mean rate of gene flow among populations from mainland and LHI populations

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Population size and splitting time

Following the running of 400,000 iterations, the DIYABC program indicated through its outputs that the most logical scenario based upon the historical model and the additional data input was scenario 1 (Figure 5) (Cornuet et.al, 2014). The model worked under the assumption that one generation occurs every five years.

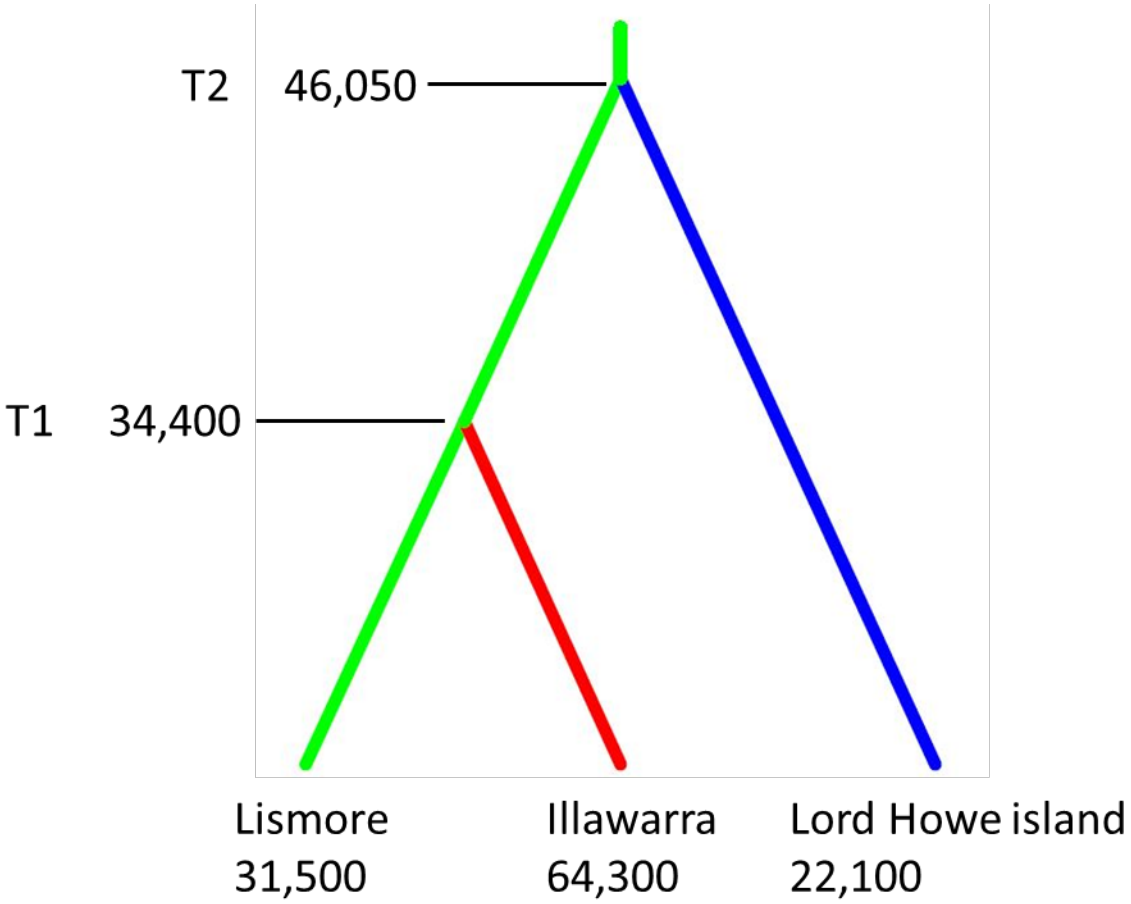


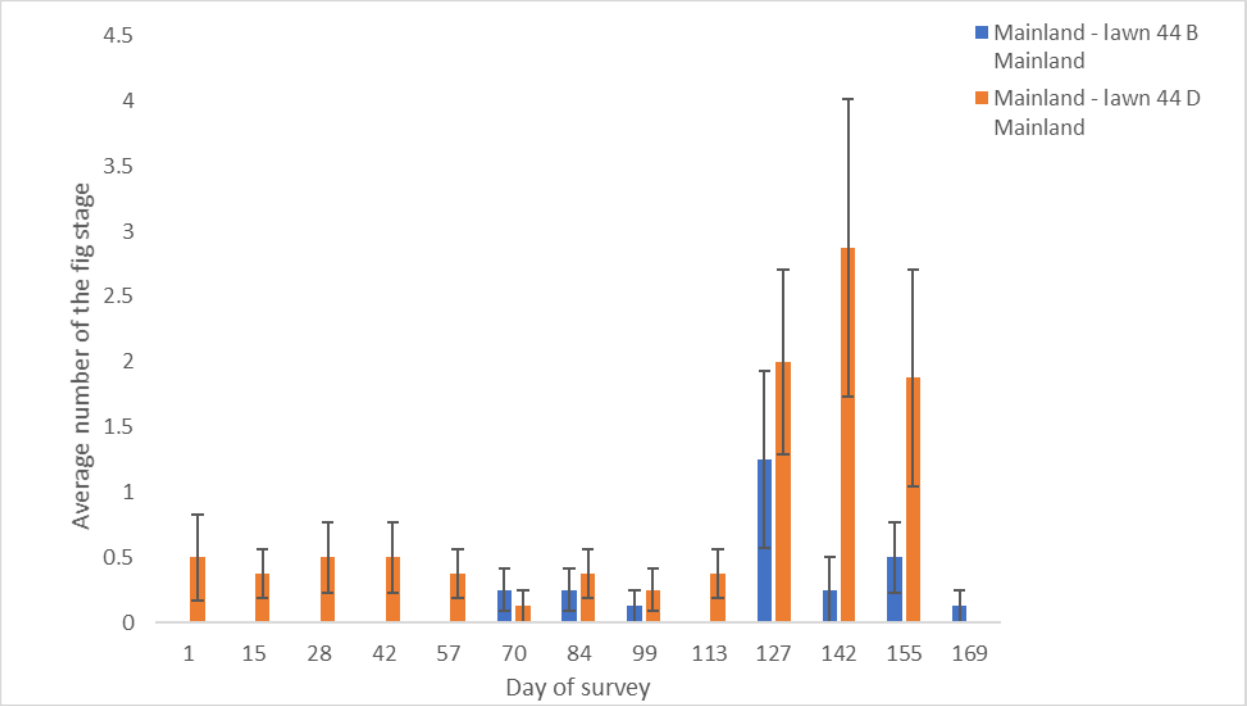
Figure 5 DIYABC Scenario 1

Flowering phenology

The results from the phenological surveys performed on Moreton Bay fig trees shown in Figure 4 highlight that it is possible for hybridisation to occur between the two forms due to the simultaneous availability of developmental stages B (receptive fig

for pollination) and D (release of fig wasps with pollen). In addition, the phenological surveys have shown that the mutual interaction between the fig tree and the pollinator fig wasp seem to thrive under warmer conditions rather than colder conditions as seen across all four locations throughout the peak in flowering during the spring and summer seasons.

Consequently, the surveys undertaken provide concise evidence that the potential for hybridisation without the association of human activity is possible. However, for naturalised hybridisation to occur the Moreton Bay fig of both forms will require to be in close proximity for the fig wasp to deposit the pollen into a receptive fig.



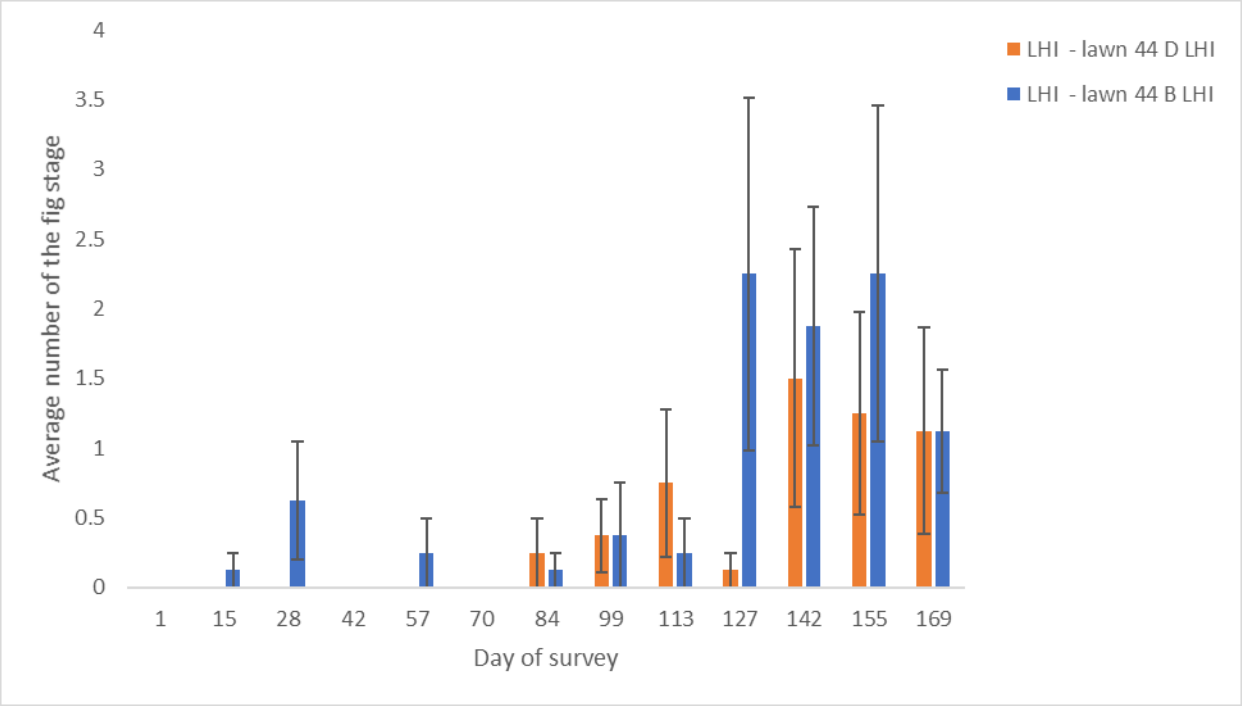


Figure 6 Average presence of fig fruit at developmental stages B and D

Seed viability

The germination of fig seedlings from fertilised fig fruit sampled from the Royal Botanic Gardens and Lord Howe island showed varied between populations. Not all the fig fruit planted germinated while other had multiple seedling germinate from the same fig fruit. In addition, the mainland had an average of 8 +/- 1 SE, the Lord Howe island (Royal Botanic Gardens) 12 +/- 2 SE and the Lord Howe island 2 +/- 0 SE.

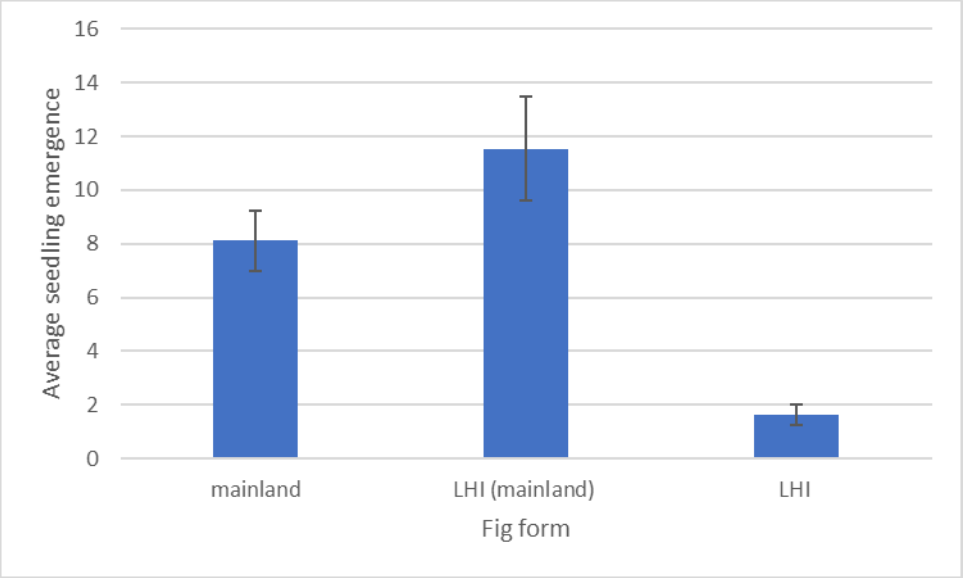


Figure 7 Average emergence of fig seedlings

Discussion

From the data presented, three of the five hypotheses formulated were supported. These hypotheses were: **greater genetic diversity in the mainland populations than in the Lord Howe island population; mainland and Lord Howe Island populations are genetically different; and reduced gene flow between the mainland and Lord Howe island populations.** The reason to support these hypotheses were that the Lord Howe island form is genetically distinct from the mainland with reduced gene flow and genetic diversity. However, the Moreton bay fig had moderate levels of inbreeding and phenological overlap within trees that was similar across populations.

On the other hand, the remaining two hypotheses were rejected (or not supported). These hypotheses were: **Lord Howe island has reduced effective population size with greater inbreeding compared to mainland populations; and reduced**

phenological overlap among the mainland and Lord Howe Island forms compared to overlap of reproductive stages within forms. The reason to not support these hypotheses was there is a shared peak flowering time for both mainland and Lord Howe island form and viable seed production of the isolated Lord Howe island tree planted in the Royal Botanic Gardens, Sydney. This gives an indication of cross compatibility and potential hybridisation.

As a result, these findings have implications for how the theory of biogeography and the concept of speciation is understood in relation to the Moreton bay fig system. Along with the consequences of conservation of the endemic Lord Howe island form.

Island Biogeography

The *F. macrophylla* population on Lord Howe Island had low levels of gene flow with the Australian mainland populations which was genetically differentiated from Lord Howe island. The Lord Howe island population had low diversity, low effective population size and lower expected heterozygosity in comparison to the mainland populations (Table 2). In contrast, to the mainland populations that showed greater expected heterozygosity, effective population size and diversity among the three populations (Table 2). However, the inbreeding coefficient indicated there is non-random mating occurring and therefore is evidence for partial self and/or biparental breeding (F = 0.24 – 0.32) in all populations. The overall level of gene flow indicates it occurs most predominantly from north to south on the mainland while the rest of the gene flow interactions between the populations occur at an order of magnitude lower (Figure 4).

The size of the Lord Howe island greatly impacts the genetic structure of the *Ficus macrophylla columnaris* population. This is because the greater the effective population size, the better genetic diversity within the population is. As a result, it would be expected the population structure of the *Ficus macrophylla columnaris* population would see changes to the observed and unbiased expected heterozygosity and the inbreeding coefficients. Furthermore, the distance between the mainland and Lord Howe island affects the genetics of the two Ficus populations via geneflow. The oceanic barrier between the two landmasses and the low rate of genetic exchange shown in the BayesAss analysis (Figure 4), have resulted in alterations to the genetic structure and diversity on Lord Howe island and on the Australian mainland. Consequently, the effect of these processes on the

demographics on both Lord Howe island and the Australian mainland have shown the populations to be genetically distinct by the Structure and Discriminant analysis of principal components (DAPC) analyses (Figure 5) however due to the two forms having compatibility with same wasp pollinator and the lack of differentiation between the two forms could be the result of the founder effect when the *Ficus macrophylla* first established on Lord Howe island. As a result, a limited number of mutations have occurred within the Lord Howe island population since establishment to result in a taxonomic redescription of form rather than sub-species as highlighted by Dale Dixon (2001).

Speciation

Currently there are two forms of *Ficus macrophylla* which were taxonomically re described by Dixon (2001). As a result, the concerning question is, when is the *Ficus macrophylla columnaris* form likely to permanently separate from the *macrophylla* form?

From the data collected during the phenological surveys it is illustrated that both forms during October to December share a similar flowering phenology with a large overlap in the release of fig wasp pollinators and the availability of receptive figs for pollination, which suggest that the possibility of hybridisation but also self-pollination was possible (Figure 6). However, as these surveys were done on the Australian mainland with trees established at the Royal Botanic Gardens, Sydney. A reciprocal experiment would need to be done to see if these results are the same or whether certain abiotic or biotic factors affect the phenological cycle of the fig tree on Lord Howe island. In addition, literature published by Wieslaw Babik et.al (2015) highlighted their study organism the Howea palms grew in different soil and altitude conditions. In light of this information it could therefore be hypothesised that the soil

could have an affect on the flowering times of the fig trees on Lord Howe Island. Furthermore, the data obtained from the growth of fig seedlings from seed also indicated that fig seed from Lord Howe island germinated less than the *columnaris* and *macrophylla* seed collected from the Royal Botanic Gardens, Sydney. This would suggest the climatic and soil conditions were not optimal for the seed from Lord Howe island, however as these seed were being transported back to the Australian mainland, the seed were required to be dried in silica to avoid contamination and consequently the seed may not have been at their optimal state when they were planted into the pots. Therefore, the overall data collected from this project would indicate that the Ficus macrophylla species has experienced an allopatric speciation.

The phenology data provides support for the possible hybridisation between the two forms. Thus, concerns remain with potential hybridisation and maintaining current genetic diversity on Lord Howe island populations by the local government is an important outcome. Preventing further introduction of *Ficus macrophylla* onto the island and affecting the genetic diversity of the *columnaris* population is still a concern and should be minimised.

Potential for genetic contamination

The experiments performed make it clear that genetic contamination can affect the genetic diversity of the respective *Ficus macrophylla* populations. Currently, due to human activity there are fig trees from the mainland population established on Lord Howe island and vice versa. Overcoming the historic, oceanic barrier to natural gene flow. Furthermore, the effects can be seen in Figure 6 which are the results of the phenological surveys taken over a period of 24 weeks. In particular, over the period from October to December the data showed the greatest overlap of stages B and D between the two forms which indicated the possibility for hybridisation or self-pollination due to the proximity of the trees surveyed. Therefore, the potential for genetic contamination the mainland form has on the population on Lord Howe island has resulted in the local government having policies that entailing how to manage weeds and invasive species on the island (Lord Howe Island Board, 2016). As a result, of these policies by the local government and the oceanic barrier between the mainland and the island are currently the most effective management strategies.

Management Implications

From the data presented in Figures 6 and 7 for flowering phenology and seed viability it is evident that the two *Ficus macrophylla* forms require maintained segregation to prevent hybridisation and self-pollination to ensure sustainable genetic diversity of both populations. As mentioned in the previous paragraph currently the only known current management strategies to prevent genetic contamination between the two *Ficus macrophylla* forms. However, as there are various modes of dispersal apart from the fig wasp and human cultivation for ornamental purposes that can transport the pollen and seed of Ficus macrophylla, it is clear the oceanic barrier

is an important natural management strategy along with the local government policy are effective in preventing larger scale hybridisation events between the two forms.

Future investigation and limitations

Further investigations that could be done in the future would be: in understanding the consequences of a reduction or loss of the fig wasp (sustainability of the fig wasp population on Lord Howe island); do soil types affect the germination and growth rate of figs? Will climate change cause the phenology of the *Ficus macrophylla* to occur earlier? Genetic confirmation of hybrid seed

Limitations that occurred throughout this project were: Using a sample design created by another person which can limit the total number of individual samples able to be used per population. In addition, having to use a small dataset to extrapolate information to infer and apply theoretical concepts such as the island of biogeography.

Conclusion

Following the outcomes of the project it is clearly evident that the genetic diversity and the population demography of the two *Ficus macrophylla* populations have confirmed what was expected prior to performing the experiments. However, the phenological and seed viability of the two forms in comparison lacked lengthy data collection such as with the surveys to be assertive with the certainty of the outcomes of these experiments. Consequently, this can affect potential decisions for applications of possible new management strategies or revision of current ones. Another consequence of this research is providing the general public as well as the Lord Howe island community the importance this species has to the Australian ecosystem.

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